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AWARD NUMBER: DAMD17-03-1-0149

TITLE: Immunomodulation of Hyperthermia for Recurrent Prostate Cancer

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REPORT DATE: March 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
March 2006	Final	1 Mar 03 – 28 Feb 06

Immunomodulation of Hyperthermia for Recurrent Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0149

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Montefiore Medical Center
Bronx, New York 10467

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

There is currently no curative treatment for recurrent hormonally refractive prostate cancer (PC). Radiation therapy (RT) has been used successfully in the treatment of primary PC, however it is not used to treat locally recurrent PC because of the high morbidity of additional pelvic irradiation and the radiation sensitivities of adjacent normal tissues. Hyperthermia has undergone extensive study as a treatment for BPH and its safety is very well established. Our goal is to develop a treatment strategy utilizing hyperthermia as an adjunct tumoricidal therapy for recurrent PC. We propose an in situ tumor vaccination approach where irradiated/heated tumor cells release peptides/antigens following localized treatment while in vivo cytokine-stimulated dendritic cells (DCs) or intratumoral injection of in vitro derived autologous DCs (bone marrow generated) harvest these antigens resulting in an effective tumor-specific immunity. This proposal has the following specific aims- Specific Aim I. To determine whether DCs are activated following antigen uptake from PC cells treated with hyperthermia. Specific Aim II. To determine whether DC-stimulating cytokines (GM-CSF, Flt3L and CD40L) following local hyperthermia of primary tumor induce specific immunity and improve local and distant tumor regression. Specific Aim III. To determine DC activation after antigen uptake is dependent upon preexisting microenvironment of PC.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Hyperthermia; APC's; Dendritic Cells; CD40L; GM-CSF; cytokines

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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A. INTRODUCTION

Prostate cancer is the most common malignancy in American men and the second most common cause of death. Currently there is no curative treatment for recurrent PC. Although, radiation therapy has been offered for organ-confined disease, it has failed to eradicate both local or systemic hormone refractory disease. Hyperthermia (HT) has been used as an anti-cancer modality or in the treatment of benign prostatic hypertrophy (BPH). Hyperthermia induces the expression of heat shock proteins (HSPs) that act as chaperones and bind to denatured proteins and peptides in HT-treated cells. HSPs bind to antigenic peptides, which can be presented to dendritic cells (DCs) for inductionof immune response. We *hypothesized* that prostate tumor cells treated with localized HT could potentially serve as a source of tumor antigens *in vivo*, where apoptotic/necrotic cells would release tumor antigens gradually over time. HT as a localized treatment would provide necessary "danger" signals" to release the HSPs and chaperone these antigenic peptides to DCs which are the most potent antigen-presenting cells (APCs). Additionally, it was hypothesized that after localized PC therapy by HT, immunotherapy with DC activating cytokines such as GMCSF, Flt3L and CD40L or *in situ* tumor vaccination with DC (autologous bone marrow derived) would provide an environment for tumor antigen uptake, processing/presentation to the host immune system. Thereby, a strategy of *in situ* tumor vaccination; PC cells release tumor antigens following local HT treatment, while cytokine-stimulated DCs harvest antigens resulting in an effective cell mediated immune response by the host. Our proposal has the following specific aims-

Specific Aim I. To determine whether DCs are activated following antigen uptake from PC cells treated with Hyperthermia (HT). HT was administered either as incubation in a 43.7^{o}C water bath or by High frequency focused ultrasound (HIFU)).

Specific Aim II. To determine whether DC-stimulating cytokines (GM-CSF, Flt3L and CD40L) following local hyperthermia of primary tumor induce specific immunity and improve local and distant tumor regression.

B. BODY

B.1. HT induced by water bath (43.7C,1hr) *in vivo* was compared to **HIFU** *in vivo.* HIFU induces coagulative tissue necrosis in the focal zone by rapidly elevating tissue temperature in a short exposure (seconds) while keeping the intervening tissue temperatures at physiologically safe levels. Preliminary results

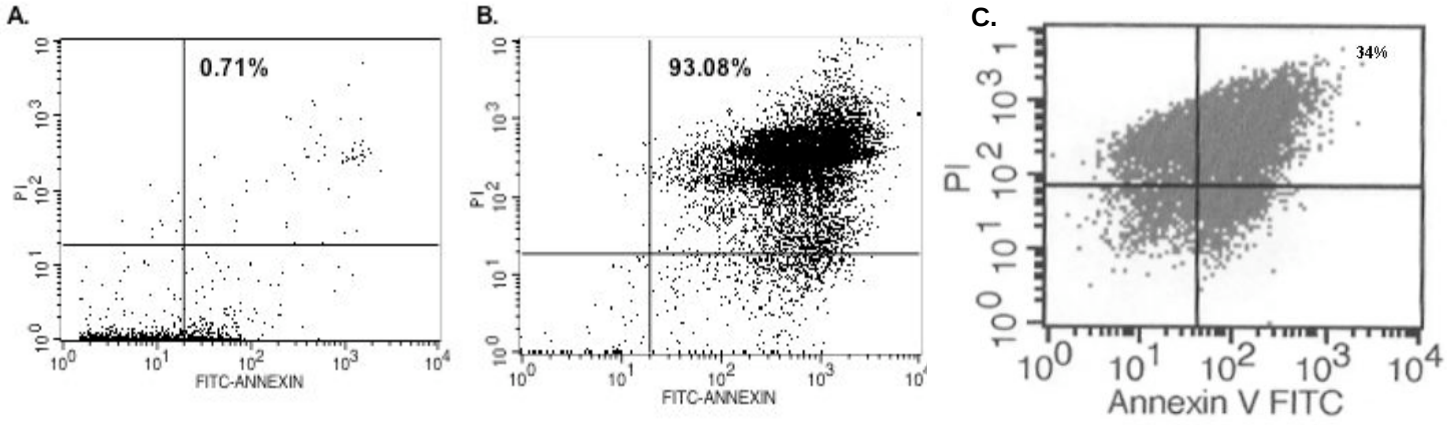


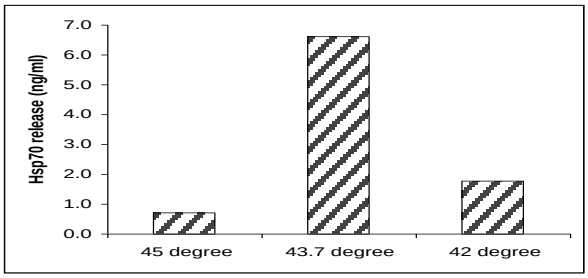
Figure 1. Hyperthermia induces apoptosis in tumor cells in vivo. Tumor bearing foot pad has been immersed in the water bath heated at 43.7C for 1 hr. After 24 hr cells were harvested and stained for FACS with PI and AnnexinV-FITC (A) untreated tumor, (B) 24 hr after HT treatment (1 hr incubation in 43.7^{o}C water bath) and (C) 24 hr after HT treatment (5 minutes of HIFU).

of HIFU trials in patients with organ-confined PC have demonstrated that HIFU is safe, and achieves a high rate of negative biopsy after treatment (85-93%). It can target regions deep in tissue without affecting intervening tissues, be applied from outside the prostate repeatedly and does not require sterilization or insertion of instruments into the gland. Additionally, HIFU has low morbidity with few complications but more importantly, localized prostate HT by water bath modality is not adequate at depth for clinical application in PC.

Hyperthermia induces apoptosis of tumor cells and the expression and release of HSPs. PC (RM-1) treated at 43.7C for 1 hour (water bath) (Fig 1B) or HIFU (60C for 5min) (Fig 1C) heat-treated tumors (thermocouple thermometry) apoptose/necrose after HT. To determine whether hyperthermia (HT) treatment of tumor in vivo induces apoptosis/necrosis, RM-1 tumors were grown on the dorsum of foot of C57Bl/6 mice. Two to 3weeks old RM-1 tumors (350mg) were treated with HT (43.7°C for 1 hr) or HIFU. At various time points, the tumors were excised and tumor cells were harvested for FACS analysis after PI/Annexin V-FITC staining. One day after HT, there was significant increase in apoptotic cells in HT-treated RM1 tumors (90±5%) (Fig.1B and 1C), compared to untreated controls (<1%) (Fig.1A).

These experiments suggested that HT treatment (water bath or HIFU) of tumors could provide a source of tumor-derived HSPs and antigens that are released from HT-treated dying tumor cells.

A.



B.

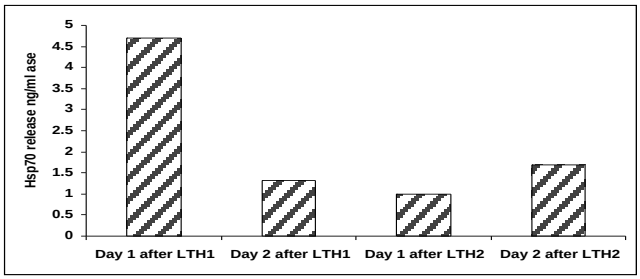


Figure. 2. Release of Hsp70 in vitro after hyperthermia treatment at different temperature. **Figure 2A.** RM1 cells grown in T-25 flask were immersed in a water bath and heated for 1 hr at 42C, 43.7C, and 45C separately. 24 hr after hyperthermia treatment cell lysates were prepared and assayed for HSP70 expression by ELISA. **Figure 2B.** HT induced release of Hsp70 in the serum. Tumor bearingfoot pad has been immersed in the water bath heated at 43.7C for 1 hr. Localized tumor HT (2 doses separated by 5 days). First dose of localized hyperthermia is indicated by LTH1 and 2nd dose of localized hyperthermia is indicated by LTH2

B.2. DCs are activated following antigen uptake from PC cells treated with hyperthermia. Autologous DCs were prepared from bone marrow derived cells after *in vitro* co culture with GMCSF and IL-4 according to published methods. Previously we have shown that immature BM DCs can engulf flurochrome labeled hepatocellular carcinoma cells (HCC) and after engulfment efficient maturation signals are provided for maturation. DCs induce the expression of cell surface costimulatory molecules (CD80/CD86). Immature BM derived DC can mature (CD11c+ CD80+) after engulfment of HT treated RM1 cells and express costimulatory signals for T-cell activation. HT-treated RM1 cells (43.7°C for 1 hr.) or untreated RM1 cells were co-incubated with immature BMDC. FACS analysis demonstrated that there is only 10% increase in CD80+ DCs when they are co-incubated with untreated RM1 cells (Fig 3A). In contrast, when incubated with HT-treated RM1 cells, there is a 31% increase in CD80+ DCs (Fig 3B).

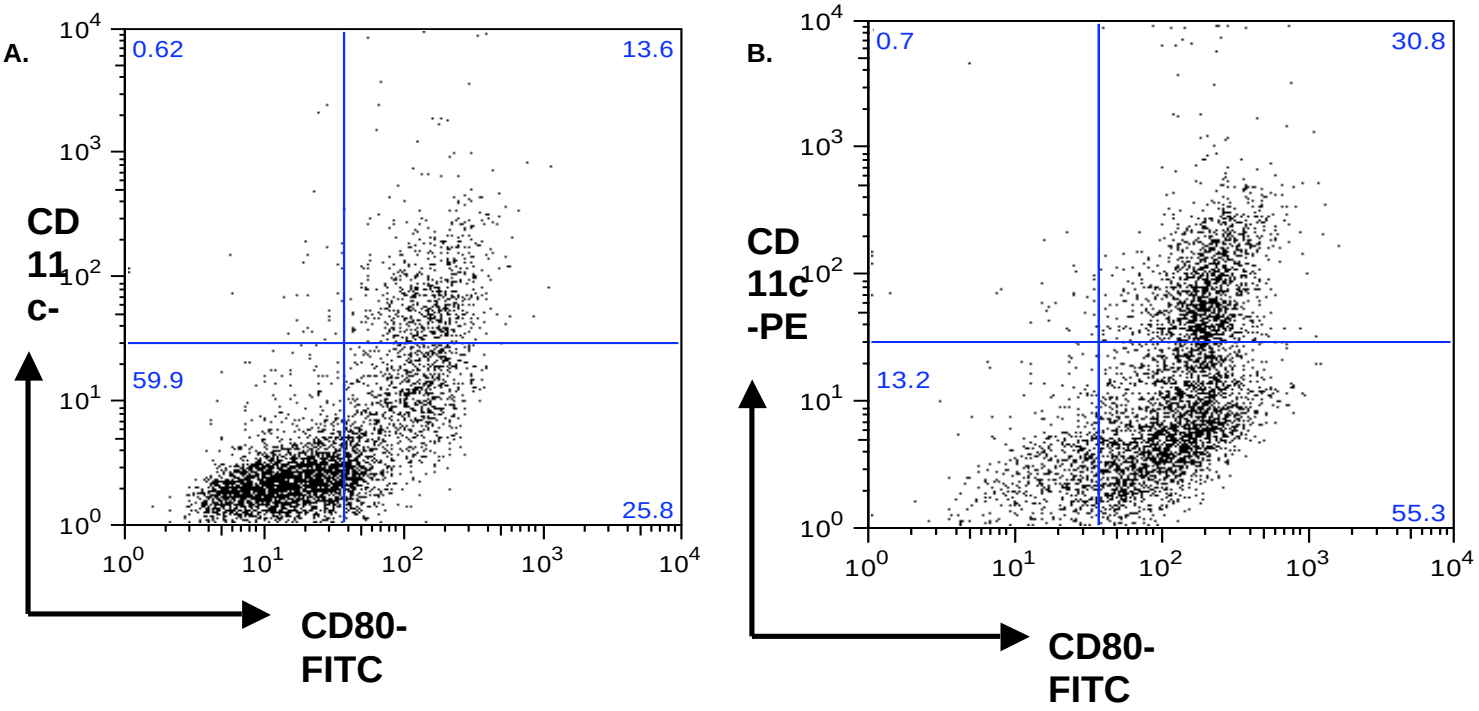


Fig. 3. After engulfing heat treated RM1 cells DC showed more maturation signal in terms of CD80 surface expression. 24 h after coincubation of DC with (**A**) untreated, (**B**) heat-treated RM1 cells.

B.3. HIFU treatment of RM-1 tumors induces activation of T helper subtype 1 (TH1) response. *We hypothesized that CD4+ve T helper cells would be activated in response to antigen presentation by DCs that engulfed HSPs and antigenic lysates from HIFU-treated tumor cells*. A marker for TH1 cell activation is increase in number of interferon gamma (IFNγ)-producing T cells. IFNγ ELISPOT assays were, therefore, performed using splenocytes isolated from C57Bl/6 mice with RM-1 tumors that received HIFU treatment. Three regimens of HIFU treatment were used in these experiments: 1) High HIFU (60^0C for 5 min); 2) Low HIFU (45-50^0C for 5 min) and 3) Low HIFU, followed by High HIFU, 2 days after the 1st HIFU treatment. The last cohort examined whether induction of HSPs by a sub-lethal low-dose HIFU, followed by a lethal high-dose HIFU resulted in increased antigenic stimulation to CD4+ve TH1 cells. ELISPOT assays demonstrated an increase in IFNγ-secreting T lymphocytes in the spleen of C57Bl/6 mice with RM1 thigh tumors that were treated with low HIFU, followed by High HIFU (Fig. 4), indicating an amplification of RM-1 specific T cell immunity following exposure to HIFU-treated RM-1 cells.

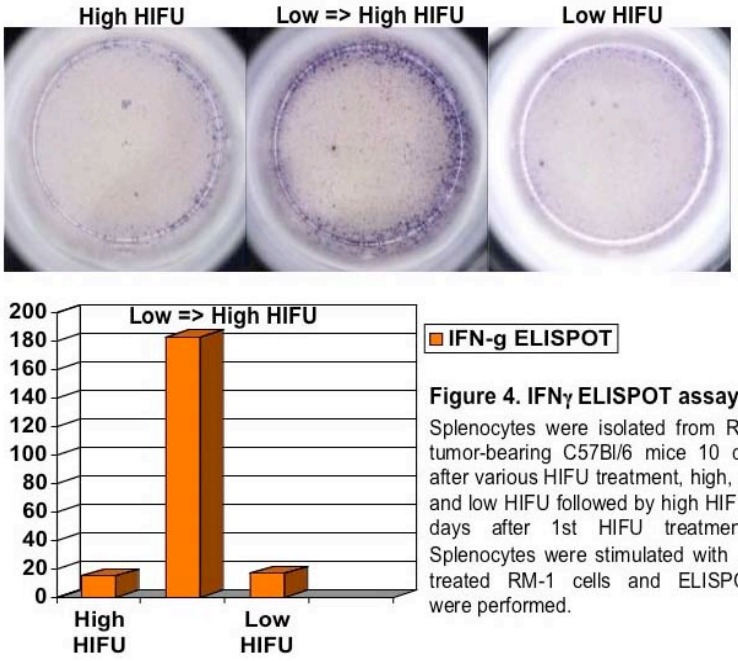


Figure 4. IFNγ ELISPOT assay. Splenocytes were isolated from RM-1 tumor-bearing C57Bl/6 mice 10 days after various HIFU treatment, high, low, and low HIFU followed by high HIFU, 2 days after 1st HIFU treatment . Splenocytes were stimulated with HT-treated RM-1 cells and ELISPOTS were performed.

B.3. HT inhibits tumor growth in C57Bl/6 mice. Mouse prostrate cancer cells, RM-1 ($1x10^5$ cells), were injected into the dorsal side of the foot of C57Bl/6 mice. Two to three weeks after tumor cell inoculation, palpable tumors were treated with hyperthermia (43.7°C) in two treatments 3 to 5 days apart. Compared to control hyperthermia inhibited the tumor growth (Fig. 5 and 6).

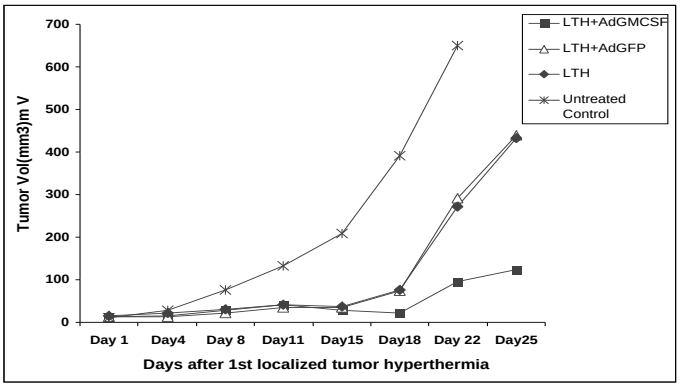


Figure.5. RM1 tumors were treated with HT (43.7C for 1 hr) on Day 0 and Day 4. AdGMCSF/AdGFP ($8x10^9$) given intra tumor (i.t.) on Day 1.

B.4. Intratumoral DC injection following hyperthermia showed significant tumor growth delay. Three-week old RM1 (approx. 100mg) tumors, grown in the dorsum of foot of C57BL/6 male mice, were subjected to HT (43.7°C for 1 hr) in two doses separated by 3 to 5 days. Following second HT treatment, animals received either PBS or DCs (10^6 per injection) intratumorally every three days for three injections. After 1st hyperthermia treatment recombinant adenovirus secreting murine GMCSF ($8 x10^9$) was given i. v. through the tail vein in separate cohorts. Control animals received adenoLacZ or adenoGFP. RM1 tumors were followed by volume measurement. It was observed that HT alone (n=10) was responsible for tumor growth delay when compared to untreated controls (Fig.5, 6). Systemic adminstration of Adeno-GM-CSF augmented the effects of HT **(Fig 5)**, further substantiating the cytokine activation of host DC.

Compared to animals treated with HT alone, GMCSF+ itratumoral DC (it-DC)-treated animals exhibited significant (p=0.007) tumor growth delay **(Fig 6, 7)**. Administration of AdGMCSF (n=12) further enhanced the tumoricidal effects of HT+it-DC (p<0.001) (Fig.7). Systemic injection of control adenoviruses (LacZ and GFP) did not alter tumor control of HT-treated animals (p=0.67). This indicated that it-DC enhanced the tumoricidal effects of HT, which was further enhanced by systemic administration of adeno-GM-CSF.

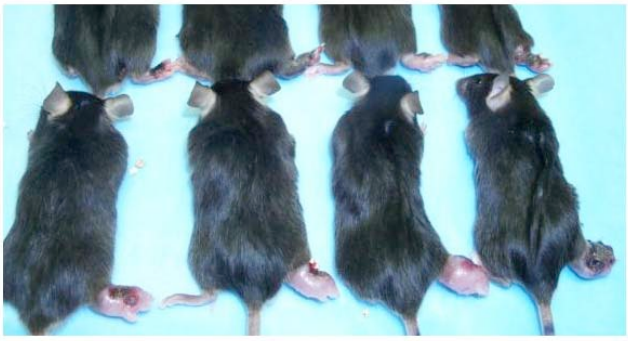
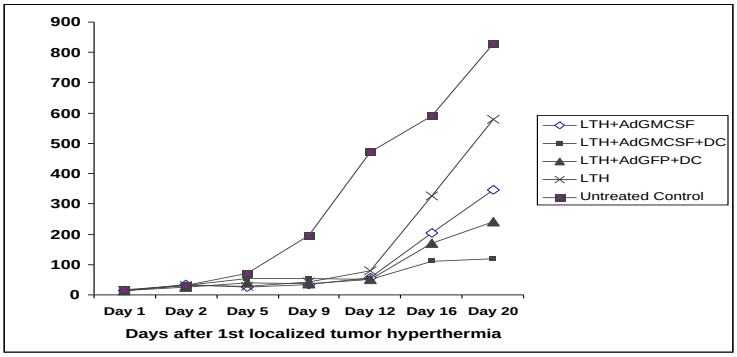


Figure. 6 Photomicrograph of RM-1 tumors. Lower row, Tumors treated with HT (43.7C for 1hr) at Day 0 and Day 4. **Upper row,** Tumors treated with HT+it-DC+Ad-GM-CSF. AdGMCSF/AdGFP ($8x10^9$) was injected i.v. on Day 1. DCs ($2x10^6$) were injected i.t. on Day 5, 8 and 11.

Fig.7. Intratumoral injection of DC increases the efficiency of tumoricidal effect of HT. Administration of AdGMCSF further augmented the effect of HT+DC.

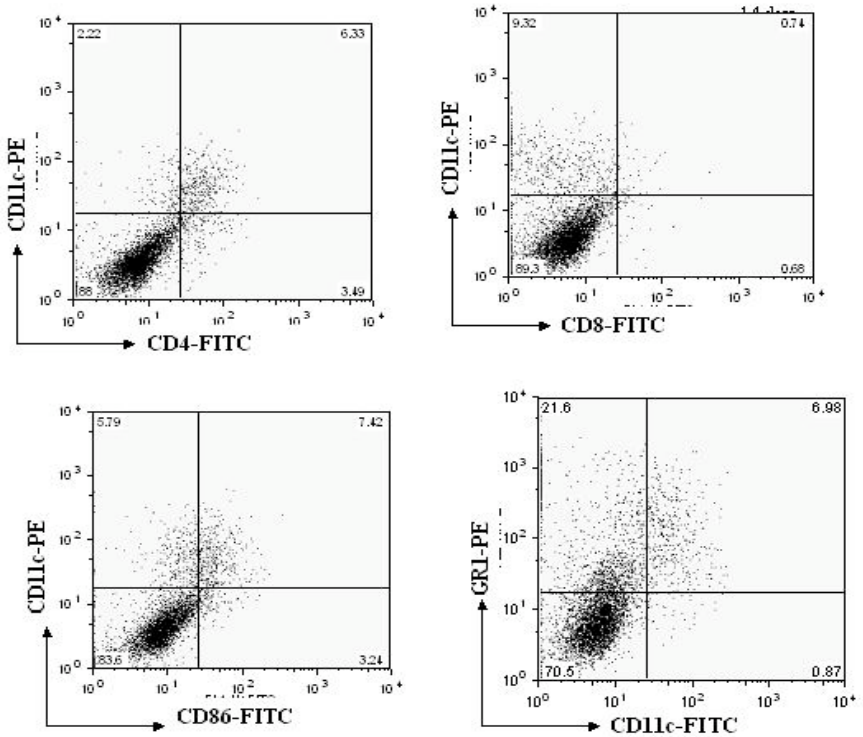


B.5. Induction of tumor-specific immunity. To evaluate whether long lived cell-mediated immunity and T cell function were implicated in the tumoricidal effects of the combination treatment, sera, splenocytes and tumor were isolated from C57Bl/6 mice, harvested at various times after HT treatment and after HT+AdGMCSF.

B.5.1. Induction of DC following adminsitration of adeno-GM-CSF

Fig 8 displays the type of DCs that are induced after HT+Ad-GM-CSF treatment. There was an increases in CD4+ CD11c+ mature DCs, as well as an increase in GR-1+ immature DCs.

Figure 8. FACscan of HT treated RM1 after systemic AdGMCSF



LTH treated tumor FACS slide (15 day after LTH and 14 day after AdGMCSF injection)

B.5.2. Induction of systemic immune response following HT and intratumoral DC + Ad-GM-CSF treatment.

A marker for TH1 cell activation is an increase in the number of interferon gamma (IFNγ)-producing T cells. IFNγ ELISPOT assays were, therefore, performed using splenocytes isolated from C57Bl/6 mice with RM-1 tumors that received HT, AdGMCSF, DC or the combination treatment. The combination therapy of HT+ AdGMCSF+ DC resulted in an increase in IFNγ-secreting T lymphocytes, indicating an amplification of RM-1 specific T cell immunity following exposure to HT-treated RM-1 cells **(Fig 9A, B)**.

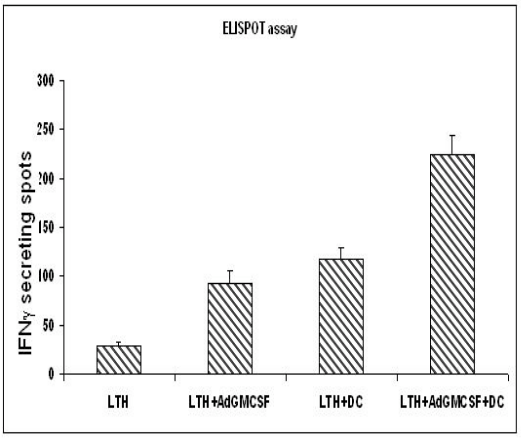
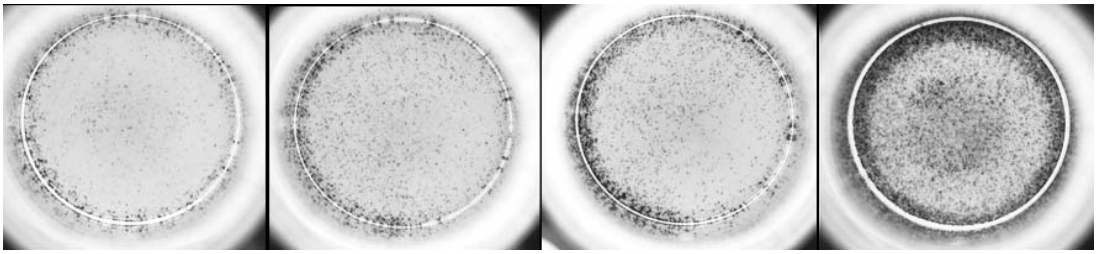


Figure. 9A. Quantitative difference in IFNγ secreting spots for various treatment cohorts in the Elispot assay (Day 22)after initial localized HT



LTH LTH+AdGMCS LTH+DC LTH+AdG MCS LTH+AdGMCS+DC

Figure. 9B. Difference in IFNγ secreting cell spots for various treatment cohorts. Elispot assay performed Day 22 after initial localized tumor HT

B.5.3. Induction of CTL response of splenocytes measured by LDH release assay

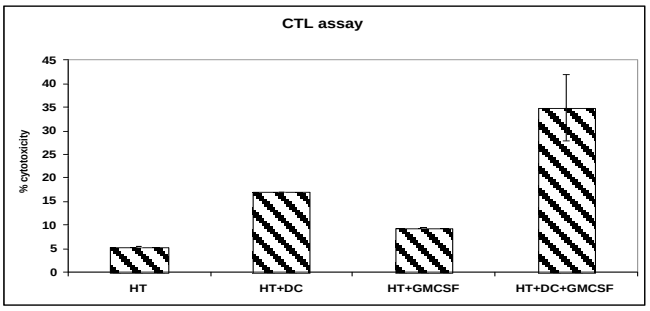


Figure. 10. CTL response in different treatment groups with an effector:target 10:1 ratio. Splenocytes were harvested (Day 22) at initial localized tumor HT, and grown (5 days with specific stimulation (heat treated (43.7C for 1 hr, RM1 cells). After 5 days CTL assay was performed using LDH release assay.

Splenocytes from immunized mice were suspended in complete RPMI-1640 containing 10% FBS and the cells were stimulated with RM1 cells; the cells were then analyzed for cytotoxic activity 5 days after in vitro stimulation. LDH-release assay was performed in a 96-well round bottom plate using RM1 cells as target cell. CTL assays were performed at lymphocyte effector: target (E:T) ratios of 10:1.

The results were expressed as: % specific analysis = (experimental release - spontaneous release / (maximum release- spontaneous release).

The LDH release assay **(Fig 10)** demonstrates that it-DC induced tumor-specific CTL activity (LDH release 17-20% vs 5% in control). AdGMCSF alone with HT has CTL activity (10% LDH release). Ad-GM-CSF augmented the CTL response to 36.0% (range, 30-44%) when given in combination with HT+DC, indicating an amplification of tumor-specific immune response by GM-CSF as an adjuvant.

B.6. Future Experiments.

B.6.1. Tolerance breaking to increase antitumor immunity by specific/non-specific approaches; antibody to CTLA4, α/β galactosyl ceramide, systemic cytoxan, whole body RT, uric acid analogs.

B.6.2. Amplification of DC maturation by CD40L and ATRA.

C. KEY RESEARCH ACCOMPLISHMENTS: **Bulleted list of key research accomplishments emanating from this research.**

- Mouse prostate (RM-1) cancer cells significantly apoptose/necrose upon HT (*in vitro* or *in vivo*) treatment when compared to other modalities (ie., high dose radiation).
- HT (43.7C 1hr or HIFU 5min 60C) significantly inhibits RM-1 tumor growth
- DCs are activated by HT or after antigen uptake by HT treated PC (RM-1) cells.
- Mature DCs induced the expression of T-cell-costimulatory molecules (CD86 and CD80) to HT PC tumor.
- Recombinant GMCSF or Ad-GM-CSF (iv) delays RM-1 tumor recurrence after HT
- Adeno β-gal or AdGFP had no significant effect in tumor growth delay over HT alone.
- AdGMCSF and DC when used in combination with HT are 2-3x more effective than either modality (GMCSF or DC with HT) for cytotoxic lymphocyte responses.

D. REPORTABLE OUTCOMES:

1) AACR poster presentation, 2005; ASTRO Translational Research poster (2) presentations 2005; ASTRO poster presentation, 2005;
2) Manuscript under preparartion; Autologous Bone Marrow derived cytokine activated Dendritic Cells confer Cell Mediated Immune Response in non-immunogenic tumors when preceeded by Localized hyperthermia.
3) Clinical need modification: Heat-treated tumors (43.7C for 1 hour (water bath) or HIFU (60C for 5min)) comparability.

E. CONCLUSIONS:

DC-based therapy (intratumoral DC injection or systemic administration of GM-CSF/FLT3L, CD40L) increases the tumoricidal effects of local tumor hyperthermia in a model of recurrent prostate cancer. DC activation (*in vitro/in vivo*) whether autologous bone marrow derived (GM-CSF activated) or by systemic GMCSF or FLT3L induces a tumor-specific cell-mediated immune response that activates both helper and cytotoxic T cells.

We have seen the induction of TH1 immune response in animals treated with HT by IFNγ ELISPOT assays demonstrating a statistically significant increase in IFNγ-releasing T cells when compared to controls. This has resulted in the induction of T cell memory as demonstrated by significant growth delays in tumor after rechallenge experiments. Attempts to amplify the HT-induced immunity (reduction of immune tolerance) with mediators of T cell inhbitory receptors/regulators (cytoxan, whole body RT, CTLA4, α/β-glactosylceramide) and inducing DC maturation (soluble CD40L and ATRA) are ongoing.

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A. Mukhopadhaya, J. Mendecki, X. Dong, A. Alfieri, C. Guha. Autologous Bone Marrow derived cytokine activated Dendritic Cells confer Cell Mediated Immune Response in non-immunogenic tumors when preceeded by Localized hyperthermia. *manuscript in prep*. Clinical Cancer Research, 2006.

G. APPENDICES:

manuscript in prep. - not attached - will provide copy when published

A. Mukhopadhaya, J. Mendecki, X. Dong, A. Alfieri, C. Guha. Autologous Bone Marrow derived cytokine activated Dendritic Cells confer Cell Mediated Immune Response in non -immunogenic tumors when preceeded by Localized hyperthermia. Clinical Cancer Research, 2006.